GLS1 expression and its role in mesenchymal stem cell aging and metabolic reprogramming

Liyang Gao#*[1,2,] Wenjing Tian*[1,2,], Chunchun Duan[1,2,], Liyang Zhu[1,2,]

1 Key Laboratory of Ministry of Education for Conservation and Utilization of Special Biological Re-sources in the Western, Ningxia University, Yinchuan, China;

2 Life Science School, Ningxia University, Yinchuan, China;

* These authors contributed equally to this work and should be considered co-first authors

Correspondence: Liyang Gao

Email: pandarun@nxu.edu.cn

Phone number: 0086-15622097174

Address: No. 539 Helanshan Road, Xixia District, Yinchuan City, Ningxia University. 750001

Authorship

Conceptualization, Liyang Gao; methodology, Wenjing Tang, Liyang Gao; validation, Liyang Gao, Wenjing Tang; formal analysis, Wenjing Tang and Chunchun Duan; data curation, Wenjing tang, and Chunchun Duan; writing—original draft preparation, Liyang Gao; writing—review and editing, Liyang Gao, Liyang Zhu; funding acquisition, Liyang Gao. All authors have read and agreed to the published version of the manuscript.

Abstract

Mesenchymal stem cells (MSCs) possess the ability to differentiate into various cell types, facilitating the repair of damaged tissues and organs while enhancing their functions, thereby playing a role in healing age-related decline. However, MSCs are also subject to aging, leading to a significant reduction in their self-renewal capacity and differentiation potential. We observed a marked increase in cell size among aged MSCs, with GLS1 expression levels in these enlarged cells being significantly higher compared to those in normal cells. Inducing cellular aging with D-galactose further elevates GLS1 expression and is accompanied by an increase in cell size. Moreover, GLS1 overexpression may enhance the expression of key glycolytic enzymes through the upregulation of FOXK2, leading to metabolic reprogramming. Conversely, overexpressing GLS1 in normal cells does not directly elevate the expression of aging-related markers, nor does it impede cell proliferation. However, GLS1 overexpression appears to diminish differentiation potential by inhibiting the Wnt/β-Catenin signaling pathway. Thus, inhibited differentiation ability of senescent MSCs, which expressed higher GLS1, may be a protective mechanism for the body to prevent the risk on newly generated tissue after the differentiation. This study investigates the relationship between GLS1 and aging, proposing that GLS1 could serve as a potential biomarker for assessing MSC quality, thereby minimizing differences in effects during MSCs-based therapy. Furthermore, elucidating the association between GLS1 and MSC senescence is crucial for advancing our understanding of the intricate mechanisms underlying MSC aging.

Keywords: Mesenchymal stem cells; cellular senescence; Glutaminase 1; glycolysis; cell differentiation; cell morphology

1 Instruction

The senescence of mesenchymal stem cells (MSCs) is characterized by a significant decline in the cell proliferation rate over time, alterations in the differentiation potential, and increased expression of senescence markers such as β-galactosidase[1]. This phenomenon poses a substantial challenge for the application of MSCs in regenerative medicine, as their therapeutic efficacy diminishes with age and prolonged in vitro culture[2,3]. These changes are critical in determining the suitability of MSCs for clinical applications, particularly in tissue engineering and cell-based therapies[4–6]. Additionally, the differentiation capacity of MSCs is diminished, and morphological changes occur, including increased cell volume and reduced mitochondrial function. Senescent MSCs also secrete higher levels of senescence-associated secretory phenotypes (SASP), adversely affecting neighboring cells[7,8]. The Wnt/β-catenin signaling pathway plays a crucial role in MSCs differentiation, and self-renewal; therefore, any abnormalities within this pathway can impair MSC function[9–12]. For instance, decreased stability of β-catenin or inhibition of upstream Wnt signaling molecules results in β-catenin's failure to translocate to the nucleus, where it typically interacts with transcription factors to activate gene transcription. This impairment consequently leads to reduced proliferation and differentiation capabilities of MSCs and promotes their senescence[3,13]. As a result of diminished proliferation and differentiation, senescent MSCs struggle to facilitate effective tissue repair[14,15]. In the context of fracture healing, senescent MSCs are less capable of differentiating into osteoblasts, which may lead to delayed or incomplete bone repair[13,16,17]. Similarly, during conditions such as myocardial infarction, senescent MSCs provide insufficient support for myocardial cell regeneration, adversely impacting recovery of heart function[18,19]. Furthermore, in the realm of immunomodulatory cell therapy, senescent MSCs exhibit increased secretion of inflammatory factors, which not only fails to mitigate excessive immune responses but may also exacerbate the activation of immune cells[4,20].

The MSCs exhibit heterogeneity, evident in variations in gene expression and differentiation potential[21,22]. These cells can differentiate into various lineages, including osteoblasts, adipocytes, chondrocytes, cardiomyocytes, and vascular endothelial cells. Even MSCs derived from the same source may show distinct differentiation preferences due to their gene expression profiles. Additionally, the differentiation potential of MSCs may show a heterogeneous way due to cellular replicative senescence and asymmetric division. Recent studies have employed single-cell sequencing to fully analyze the heterogeneity of MSCs[23–26]. Through data retrieval and analysis, we noticed a subset of MSCs with significantly elevated expression of Glutaminase 1 (GLS1). Furthermore, in our experiments involving D-galactose-induced cellular senescence, we also observed an increase in GLS1 expression in treated UCMSCs.

Some experiments have established a connection between high expression levels of GLS and senescent cells[27,28]. GLS catalyzes the hydrolysis of glutamine into glutamic acid and ammonia. It serves as a critical enzyme in glutamine metabolism, contributing to various essential intracellular functions, including serving as a source of nitrogen and carbon in biosynthetic processes.

Researches have explored the relationship between GLS and cell senescence, including cancer cell senescence[27], somatic cell senescence[28], hematopoietic stem cell senescence[29,30], and embryonic stem cell senescence[31]. For example, the inhibition of GLS1 expression in cancer cells

has been found to increase oxidative stress, thereby inducing senescence[32,33]. In senescent somatic cells, high GLS1 expression can reduce oxidative stress by facilitating glutathione (GSH) synthesis, while also compensating for the energy deficits resulting from impaired mitochondrial function through enhanced glutamine metabolism, ultimately supported the survival of senescent cells [34,35]. Conversely, inhibiting GLS1 activity may lead to the elimination of senescent somatic cells[36,37]. In hematopoietic stem cells, GLS1 provides essential energy support through glutamine metabolism, which is crucial for maintaining their normal function and preserving intracellular redox balance via GSH synthesis. Inhibiting GLS1 activity may result in the senescence of hematopoietic stem cells. Similarly, during embryonic stem cell differentiation, GLS expression is dynamically regulated by cellular signals, highlighting the effect of modulating glutamine metabolism during cell differentiation.

Additionally, GLS1 has been found to support the survival of senescent umbilical cord-derived mesenchymal stem cells (UCMSCs) by promoting cell division. However, there is currently insufficient experimental evidence to reveal whether elevated GLS1 expression is a crucial factor in MSC senescence. Therefore, discovering the relationship between GLS1 and MSC senescence is necessary for enhancing our understanding of the complex mechanisms underlying MSC senescence and may establish an experimental foundation for future strategies aimed at improving MSC functionality through the regulation of GLS1 activity.

2 Methodology

2.1 Cell culture and transfection

We collected human umbilical from healthy donors by cesarean delivery performed after 37 weeks or more of gestation with approvement signed by the Ethic Committee (Ref. PJ2015014). human umbilical samples were transported on ice, and the processing time was less than 1h. Cell line authentication (Short tandem repeat, STR) was used to confirm the cell lines (profiling by G&C Biotech, Shanghai). The hUCMSCs were maintained using basic DMEM (Gibco, USA) with 15% FBS (Gibco, USA). In order to rule out that cell senescence is caused by the culture medium itself, hUCMSCs also cultured using a serum-free medium (TBD, China), to confirm the effects. The cells were cultured in an incubator at 37 ℃ with 5% CO_2, and collected for subsequent experiments after 95% cell confluence. The pIRES2-Zsgreen1-GLS1 and negative controls were purchased from Universe Gene Technology (Tianjin, China). The sequence of plasmid was confirmed by DNA sequencing. The transfection started whence the confluence of cells reached about 60-80% one day after inoculation in 60mm dish. Then Opti-MEM (Thermofisher, USA) and Lipofectamine 2000 transfection reagent (Yeasen, China) were mixed according to the ratio in 10μL to 490μL and left to stand for 5 min at RT. Meanwhile, 1μg plasmid and 500μL Opti-MEM were mixed and left to stand for 5 min at RT. Next, the above two mixtures were mixed evenly and left to stand at room temperature for 15 min. Finally, the final mixture was dropped into the dish drop by drop and gently shaken, and then continued to be cultured in the incubator. After 6 hours of transfection, changed the medium with prewarmed fresh complete cell culture medium.

2.2 β-galactosidase senescence staining

The senescence β-galactosidase staining kit (Beyotime, China) was used to detect senescence cells in culture. Cells were cultured in a 35mm culture dish until research 80% confluence, then adherent cells washed with PBS (Servicebio, China), following by fixing and then washed with PBS three

times. Finally, the staining working solution was added and placed in a 37°C incubator to incubate overnight. On the next day, the staining working solution was discarded, and bluish-green cells were observed under an inverted microscope, which were the senescent cells.

2.3 Cell viability assay and ATP assay

Digest the cells of the control group and the treatment group with 0.25% trypsin (Gibco, USA), and re-inoculate them into 96-well plates at a density of 1×10^4 cells/well. On the day of experiment, add 10 μl of CCK-8 solution (Beyotime, China) to each well and incubate at 37°C for 2h-4h. After the incubation time is over, measure the absorbance at 450 nm with a microplate reader. CellTiter-Lumi kit (Beyotime, China) was used to detect the ATP level in cells. Before assay, cells were counted and replated on 96-well-plate at a density of 1×10^4 cells/well. The luminescence was measured 10 min after mixing kit with cell culture medium at ratio of 1:1.

2.4 Immunofluorescence staining

When the confluence of cells reaches about 80%, rinse with PBS, then fix with 4% paraformaldehyde solution (Servicebio, China) at RT for 20min and wash the cells with PBS three times. Next, permeabilize cells with 0.3% Triton X-100 () for 15min. After washing, block the cells with 3% bovine serum albumin (Servicebio, China) at RT for 30min. Subsequently, incubate the cells with KGA/GAC antibody (1:1000?) and β-CATENIN antibody () at 4°C overnight, respectively. TRITC-conjugated phalloidin (Yeasen, China) was used for staining ACTIN filaments. DAPI solution (Servicebio, China) was used for staining nucleus. The MitoTracker Red CMXRos (Beyotime, China) was used for staining mitochondria in living cells. JC-1 Mitochondrial membrane potential assay kit () was used to examine the condition of mitochondria inside cells.

2.5 RNA sequencing and RT-qPCR

The total RNA isolation kit (Tiangen, China) was used to purified mRNA from cells. The mRNA expression for both treated samples and control samples was profiled using RNA sequencing (RNA-Seq, Majorbio, China), with three biological replicates with each group. The PrimeScript RT reagent kit and SYBR Green PCR kit (Takara, Japan) were used for RT-qPCR analysis to evaluate target mRNA expression. All primers were synthesized by Sangon Biotech, China.

2.6 Western blotting

Proteins were collected using RIPA lysis buffer (Beyotime, China) and 1% PMSF (Beyotime, China), the protein concentration was quantified using a BCA protein concentration assay kit (Beyotime, China), and detected by a microplate reader. Add 6×Loading Buffer () and heat at 100°C for 10 minutes in a metal thermostatic bath, and then the protein samples were used for loading on a 12.5% PAGE gel () to separate proteins with different molecular weights. The separated proteins were transferred to a 0.22 μm PVDF membrane (). The PVDF membrane was rinsed once in 1×TBST (), then blocked with 5% skim milk powder () at room temperature for 2h, and then washed 4 times with 1×TBST. Subsequently, the membranes were incubated overnight at 4°C with the prepared KGA/GAC antibody (), β-ACTIN(), and GLB1 () antibody respectively. After that, the membranes were washed three times, and incubated with horseradish peroxidase-conjugated secondary antibody () at RT for 1h, and finally the super sensitive ECL luminescence reagent (MeilunBio, China) was dropped to visualize the signal.

2.7 Bioinformatics analysis

Single-cell RNA sequencing data (GSE157773) we were collected from GEO database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE157773). Utilizing the Seurat R package v4.0.2 and RStudio-1.1.453, the RNA-seq data from human mesenchymal stem cells

(GSM4774851) underwent filtering, normalization, dimensionality reduction, and differential expression gene (DEG) analysis. For data quality assurance, the mitochondrial RNA threshold was established at 10%, with gene expression limited to less than 2500 to exclude low-quality cells. The top40 principal components were selected after the 'RunPCA' function was applied, and these were used in UMAP to generate 2D projections, facilitating the sub-clustering of human mesenchymal stem cells.

The data of bone marrow MSCs, which from elder and young donors, was collected from GEO database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE157773). Four samples from elder donors (GSM308234, GSM308233, GSM308231, GSM308228) and three samples from younger donors （GSM308224, GSM308225, GSM308226）. All data were analyzed by Seurat R package v4.0.2 and RStudio-1.1.453. The gene ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed by DAVID.

2.8 Statistical analysis

Each biological experiment was conducted three times, and the data shown represent at least three separate experiments. Data presentation is in the form of mean ± standard deviation. Statistical analyses were executed using GraphPad Prism software version 9 (GraphPad Software Inc., La Jolla, CA, USA). The paired T-test (non-parametric) or Wilcoxon signed-rank test were used for making statistical comparison between treated and control groups.

3 Results

3.1 The GLS1 gene is highly expressed in a subset of UCMSCs that exhibit larger cell sizes

To assess the size of normal UCMSCs, we cultured passage 2 UCMSCs for 48 hours and subsequently captured phase-contrast images (Figure 1A). The area of each cell was quantified using ImageJ software, revealing that cell sizes ranged from 100 μm² to 4000 μm² (Figure 1B), with an average size of approximately 1500 μm². However, after extending the cell subculture passages, some UCMSCs displayed greater cell body sizes compared to those in earlier passages. Notably, we observed that the GLS1 protein expression level in cells exceeding 3900 μm² was significantly higher than that in smaller cells (Figures 1C-F).

To investigate the heterogeneity of UCMSCs, we analyzed single-cell transcriptome data from MSCs available in the GEO database (GSE157773). This analysis identified four subclusters among all cells, with GLS1 exhibiting high expression in group 2 (Figures 1G-I). Additionally, we retrieved RNA sequencing data from bone marrow MSCs isolated from four elderly donors and three young donors (GSE12274), and found that GLS1 expression in aging MSCs revealed a significant increasing (Figure 1J). Subsequently, we induced cellular senescence in UCMSCs using 2% D-galactose for four days. Our observations indicated a marked increase in GLS1 expression in D-galactose-induced UCMSCs (Figure 1K).

3.2 The relationship between cell aging and GLS1 overexpression

Furthermore, to investigate the effects of GLS1 on UCMSCs, we employed two strategies to enhance GLS1 protein expression: drug-induced overexpression and plasmid-mediated overexpression. These approaches allowed for a comprehensive assessment of the effects of GLS1 on UCMSCs.

3.2.1 The cell under senescence process expressed more GLS1 than normal UCMSCs

In our first approach, immunocytochemistry (ICC) images demonstrated a significant increase in GLS1 expression following prolonged treatment of UCMSCs with 2% D-galactose for 2 and 4 days (Figures 2A-C). Notably, cells exhibiting higher GLS1 expression also displayed an increase in cell size compared to those with lower GLS1 expression (Figure 2D). Additionally, senescence β-Galactosidase staining indicated an increased number of aging cells (indicated by blue coloration) following treatment (Figure 2E). Western blot analysis further revealed that the induced expression of GLS1 was associated with elevated levels of senescence-related markers, including GLB1, P21, and P16 (Figure 2F).

3.2.2 The GLS1 overexpression did not direct induce cellular aging or alter cell morphology

In our second approach, we constructed the pIRES2-GLS1-ZsGreen1 plasmid and transfected it into UCMSCs, resulting in increased GLS1 expression. The cell morphology of GLS1-overexpressing cells did not differ from that of cells transfected with the empty vector (Figures 3A-B). Confocal microscopy revealed that hUMSCs, which exhibited elevated GLS1 levels post-transfection, divided into two cells with equally high GLS1 expressions after cell division (Figure 3C). Additionally, SA-β-gal staining indicated that the number of aging cells (characterized by blue coloration) did not increase following GLS1 overexpression (Figure 3D). Western blot analysis further demonstrated that the overexpression of GLS1 did not alter the expression levels of senescence-related markers (GLB1, P21, P16) in the transfected UCMSCs (Figure 3E). Furthermore, results from the CCK-8 assay indicated that cell growth remained unaffected (Figure 3F).

3.3 The GLS1 overexpression did not impair mitochondria function but induced glycolysis related genes in UCMSCs

To assess the mitochondrial population within the cells, we stained them with MitoTracker dyes and quantified the fluorescent density using ImageJ. The results indicated no significant difference in fluorescence between control cells and GLS1-overexpressing cells (Figures 4A-B). Subsequently, we evaluated the mitochondrial membrane potential using JC-1 staining in living cells and captured images with a confocal microscope. These images revealed that nearly all cells exhibited red fluorescence, indicating intact mitochondrial membranes, while fewer green cells, indicative of compromised mitochondrial quality, were observed in both control and GLS1-overexpressing cells (Figures 4C-D). These findings suggest that GLS1 overexpression does not impair mitochondrial function.

Furthermore, we counted the same number of cells and culture them for 24 hours, followed by ATP quantification. The ATP levels in the GLS1-overexpressing group showed a slight increase (Figure 5A). Additionally, *FOXK2* mRNA levels were significantly elevated following GLS1 overexpression, along with substantial induction of its downstream genes, *HK1*, *ENO1*, and *PKM1/2*, which encode key enzymes involved in glycolysis (Figure 5B). Moreover, differentially expressed genes in GLS1-overexpressing cells were analyzed using RNA sequencing, and a network view of FOXK2-related genes was generated using Cytoscape.

3.4 GLS1 overexpression diminish the differentiation potential of UCMSCs via decreasing β-CATENIN

To investigate the effects of GLS1 on UCMSCs, we performed RNA sequencing on cells treated with 2% D-galactose and on cells overexpressing GLS1 (Figure 6A). We conducted Gene Ontology (GO) analysis on 581 genes that were significantly upregulated following GLS1 overexpression and on 611 genes that were significantly elevated in control group. The genes enriched in control UCMSCs were associated with a broader range of biological processes, including heart development, angiogenesis, osteoclast differentiation, dendrite development, head development, neurogenesis, and stem cell proliferation (Figure 6B). In contrast, the genes enriched in GLS1-overexpressing cells were primarily associated with biological processes such as angiogenesis, endothelial cell proliferation, and heart development (Figure 6C). Additionally, after treating cells with 2% D-galactose for four days, we observed that 339 genes were significantly upregulated while 295 genes were downregulated. The GO analysis indicated that the variety of enriched biological process classes was reduced compared to that of the untreated group (Figure 6D).

Then we examined the expression level of genes, which related with angiogenesis of UCMSCs. Analysis result revealed that several genes, including *VEGFA*, *WARS1*, *MMP4*, *SFRP1* and *GPNMB* were significant enriched in control group. Conversely, other genes such as *RTN4, XBP1, HMOX1, BMC10, GATA4* were significantly enriched in GLS1 overexpression cells (Figure 6E). Moreover, genes related with osteoclast differentiation, such as *SFRP1* and *CCN4,* exhibited higher expression levels in the control group. In contrast, genes related to osteoblast differentiation,, such as *CCN1*, *FGF2*, *FASN*, *TPM4,* were significantly enriched in GLS1 overexpression cells (Figure 6F).

4 Discussion

4.1 A subset of cells exhibiting high GLS1 expression during the subculturing of UCMSCs

MSCs derived from the same source can exhibit different differentiation direction and efficiencies; and some may preferentially differentiate in a specific direction. Notably, during the subculture of UCMSCs, we consistently observed the presence of larger cells with distinct morphologies compared to normal UCMSCs. Furthermore, the proportion of these larger cells increased with prolonged in vitro culture. Typically, these larger cells are considered to be uncontrollably differentiated cells, but their specific identity remains unknown, as does the mechanism underlying their increased size. Although evidences showed that cell size related with senescence and cell-cycle arrest, the profile of enlarged MSCs still not fully examined [38–40].

By analyzing single-cell transcriptomic data retrieved from GEO, a group of UCMSCs with elevated GLS1 expression was identified. In addition, we retrieved RNA sequencing data of human bone marrow MSCs from GEO, which included samples from both young and old donors. The analysis revealed that MSCs isolated from older individuals exhibited higher GLS1 expression. All these findings suggested that GLS1 may serve as a marker for aging UCMSCs, which exhibit enlarged cell bodies. This result also be confirmed by another group, who found that inhibiting GLS1 delayed the replicative senescence of UCMSCs by senolysis [36].

4.2 Aging cells exhibit larger cell bodies and elevated GLS1 expression levels.

Cell differentiation and senescence play significant roles in altering the morphology of MSCs, and cause heterogeneity by changing the gene expression profile [41]. After prolonged culture of UCMSCs, we observed that some cells increased in size. We stained these cells with GLS1

antibodies, and the immunocytochemistry (ICC) images indicated that the larger cells exhibited higher GLS1 expression compared to smaller cells. This finding was further corroborated by inducing cell senescence using D-galactose, with senescence markers GLB1, P21 and P16. These results suggest that during the senescence process, cells may experience an increase in cell area and GLS1 expression. Previous research also found the size of cells from aging mice were larger than non-senescent cells [42]. A study found mTOR promotes growth without cell division when the cell cycle is arrested by p21 and p16, resulting in cellular hypertrophy with a large morphology [43]. In our experiment, the enlarged cell bodies might also cause by increased P21 and P16 protein level.

As a key enzyme in glutamine metabolism, the activity and expression level of GLS are altered in senescent cells, implying a potential relationship between GLS and aging [37,44]. Variations in GLS activity can influence the metabolic state of cells; for instance, senescent cells typically enhance glutamine catabolism to satisfy their energy requirements and support macromolecular synthesis under stress conditions [45–47]. The upregulation of GLS facilitates glutamine breakdown, providing additional energy and metabolic intermediates for these cells. Collectively, these studies suggest that the upregulation of GLS1 benefits the energy metabolism, biosynthetic processes and survival of senescent cells.

4.3 Upregulation of GLS expression is not a direct factor in inducing cellular senescence

Abnormal GLS1 activity may result in the accumulation or deficiency of intracellular metabolites, leading to the activation of cell cycle checkpoints and consequently causing cells to enter a state of senescence-related cycle arrest. Additionally, GLS1 activity can be modulated by caloric restriction, which may inhibit excessive cell growth and proliferation by inhibiting the insulin/IGF-1 and mTOR signaling pathways, thereby decelerating replicative cellular aging [43,48]. However, in our experiment, the overexpression of GLS1 did not affect the cell cycle of UCMSCs nor induce significant alterations in the expression of cell cycle checkpoint genes. We also found that overexpression GLS1 in UCMSCs did not alter the cell morphology or the levels of senescence marker GLB1 and cell cycle arrest factor P21 and P16. These results indicate that GLS1 is not a direct trigger of cellular senescence or cell body enlargement.

4.4 GLS1 overexpression caused metabolic reprogramming and reduced cell differentiation potential

Under conditions of oxidative stress, DNA damage, or nutritional deprivation, cells induce the expression of the transcription factor FOXK2 to regulate metabolism and adapt to their environment [49–51]. We found that the increase in the expression of GLS1 would significantly upregulate the expression of FOXK2 in UCMSCs cells, and the expression levels of genes related to the expression of FOXK2 were significantly upregulated. As a transcription factor, FOXK2 can directly bind to the promoter regions of glycolysis-related genes, such as hexokinase 1 (HK1), pyruvate kinase M1/2 (PKM), and enolase 1 (ENO1), thereby influencing their transcription [51–54]. HK1 is the first key enzyme in glycolysis, catalyzing the phosphorylation of glucose to produce glucose-6-phosphate, marking the initial step of glycolysis. ENO1 is another essential enzyme in this pathway, facilitating a reaction that generates high-energy phosphate bonds. Subsequently, PKM catalyzes the conversion of phosphoenolpyruvate (PEP) to pyruvate while generating ATP, further underscoring its role as a critical enzyme in glycolysis. All these three enzymes significantly increased following GLS1

overexpression, and coherence analysis demonstrated a strong correlation with FOXK2. Therefore, we suggest that GLS1 overexpression may stimulate *FOXK2* expression by establishing cellular nutritional pressure, which subsequently enhances the expression of key glycolytic enzymes.

Previous researches reported that target GLS1 could inhibit Wnt/β-catenin signaling pathway in Hepatocellular carcinoma by increasing ROS level [55]. And GLS1 promotes p-GSK3β, which decreases β-catenin level in cell by ubiquitin proteasome mediated degradation[56]. These researches built a negative relationship between GLS1 and Wnt signaling pathway. Another report further showed FOXM1 activated nuclear translocation of β-CATENIN in glioma [57]. In our study, we discovered that the loss of the cell differentiation potential associated with a decrease in β-CATENIN in cell nucleus. However, we leaked direct evidence to show that the inhibited activity of β-CATENIN in nucleus caused by FOXK2.

Previous studies have indicated that hematopoietic stem cells (HSCs) and MSCs rely more heavily on glycolysis for their energy supply compared to differentiated cells. In this study, we observed that metabolic reprogramming did not alter the number of mitochondria or their membrane potential. Additionally, cells overexpressing GLS1 exhibited increased ATP levels, suggesting enhanced ATP production via glutamine breakdown and glycolysis. However, this metabolic reprogramming may not be advantageous for the self-renewal of stem cells. The Gene Ontology (GO) enrichment analysis and RT-qPCR results revealed that GLS1 overexpressing cells exhibited reduced differentiation potential compared to the control group, with UCMSCs losing most differentiation capabilities, except angiogenesis and endothelial cell differentiation.

5 Conclusion

During the prolonged culture of UCMSCs *in vitro*, a subset of the cells consistently exhibits an increase in cell size. Due to the limited number of these cells and the challenges associated with cell isolation, makes it difficult to ascertain the internal changes occurring within these enlarged cells. Additionally, the underlying mechanisms responsible for the increase in cell size in UCMSCs remain unclear. Notably, we have observed a significant increase in GLS1 expression in these enlarged cells. Although previous research reported that GLS1 is associated with somatic and organismal aging, and that inhibiting the activity of GLS1 may delay aging in mice, it remains uncertain whether the upregulation of GLS1 expression is a causal factor during cell aging of UCMSCs. Therefore, our study employed two methodologies: drug induction of GLS1 overexpression and gene overexpression, to investigate the effects of GLS1 on UCMSCs. We found that UCMSCs with enlarged cell bodies were senescent cells during cell subculturing, and they showed higher expression of intracellular GLS1. GLS1 overexpression might enhance the expression of key glycolytic enzymes through the upregulation of FOXK2, leading to metabolic reprogramming. Moreover, GLS1 does not directly lead to cell aging, but impaired cell differentiation capacity though decreasing the protein level of β-CATENIN in nucleus. Inhibited differentiation ability of senescent MSCs may be a protective mechanism for the body to prevent the risk on newly generated tissue after the differentiation.

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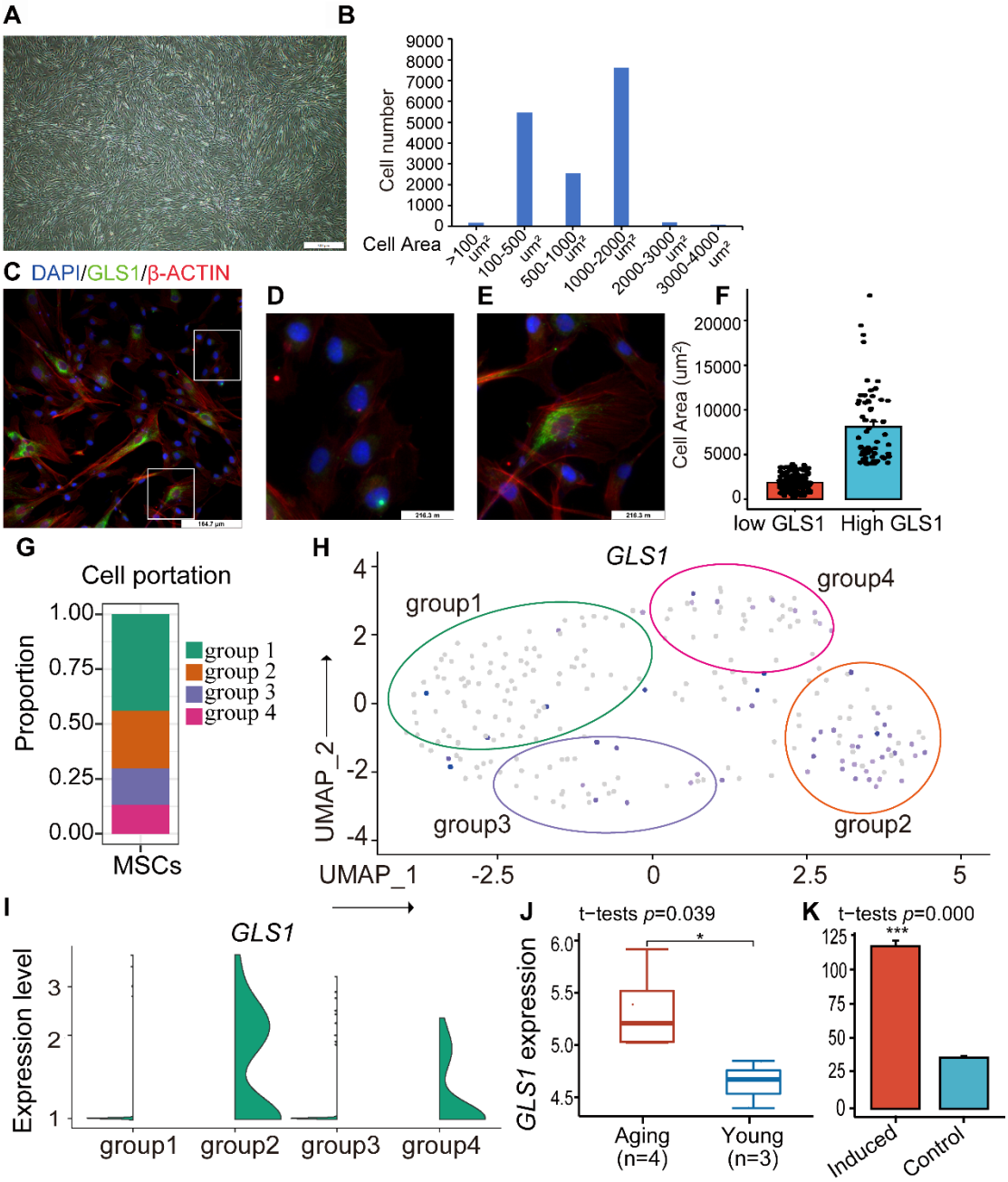

Figure 1: The morphology and GLS1 expression in UCMSCs. (A) The morphology of passage 2 UCMSCs; (B) the cell number and single cell area calculated by imageJ; (C) poor quality UCMSCs which cultured for more than 20 passages; (D) the zoom in of normal size cells; (E) the zoom in of big body cell; (F) calculated the cell size of high GLS1 expression cells and low GLS1 expression cells by using imageJ; (G) the proportion of MSCs, which devided into 4 groups by single cell analysis; (H) *GLS1* expressed in 4 groups; (I) The expression level of *GLS1* in 4 groups; (J) *GLS1* expressed in bone marrow derived-MSCs which from elder donor and young donor; (K) *GLS1* expression in D-galactose-induced cells.

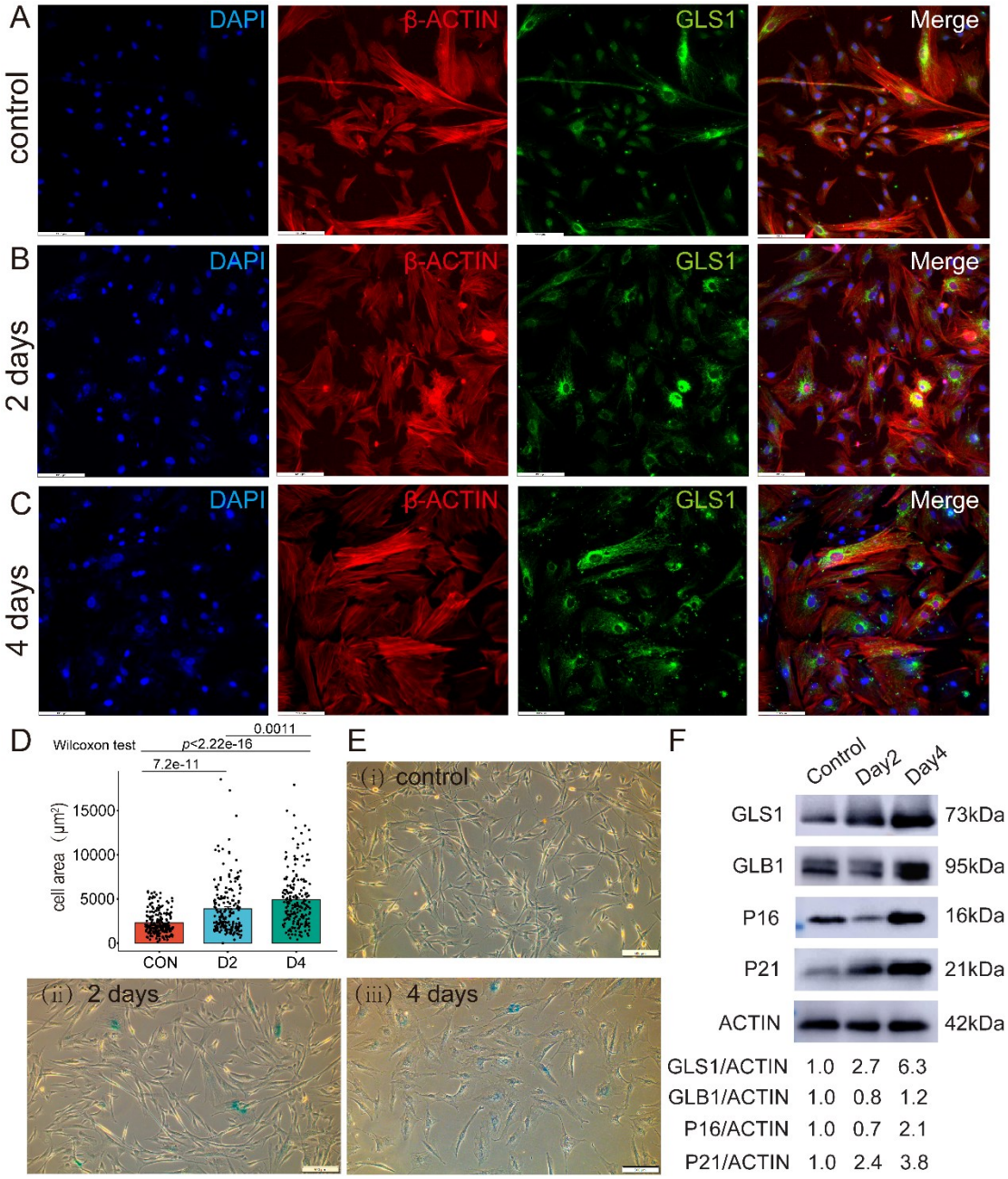

Figure 2: The GLS1 expressed D-galactose**-induced senescence UCMSCs.** The expression of GLS 1 in control group (A), 2 days D-galactose-induced group (B) and 4 days D-galactose-induced group (C); (D) the cell sizes of control cells and 2days, 4days D-galactose-induced cells; (E) senescence β-Galactosidase staining for control, 2days, 4days D-galactose-induced cells; (F) GLS1 expression and senescence-related markers in three groups.

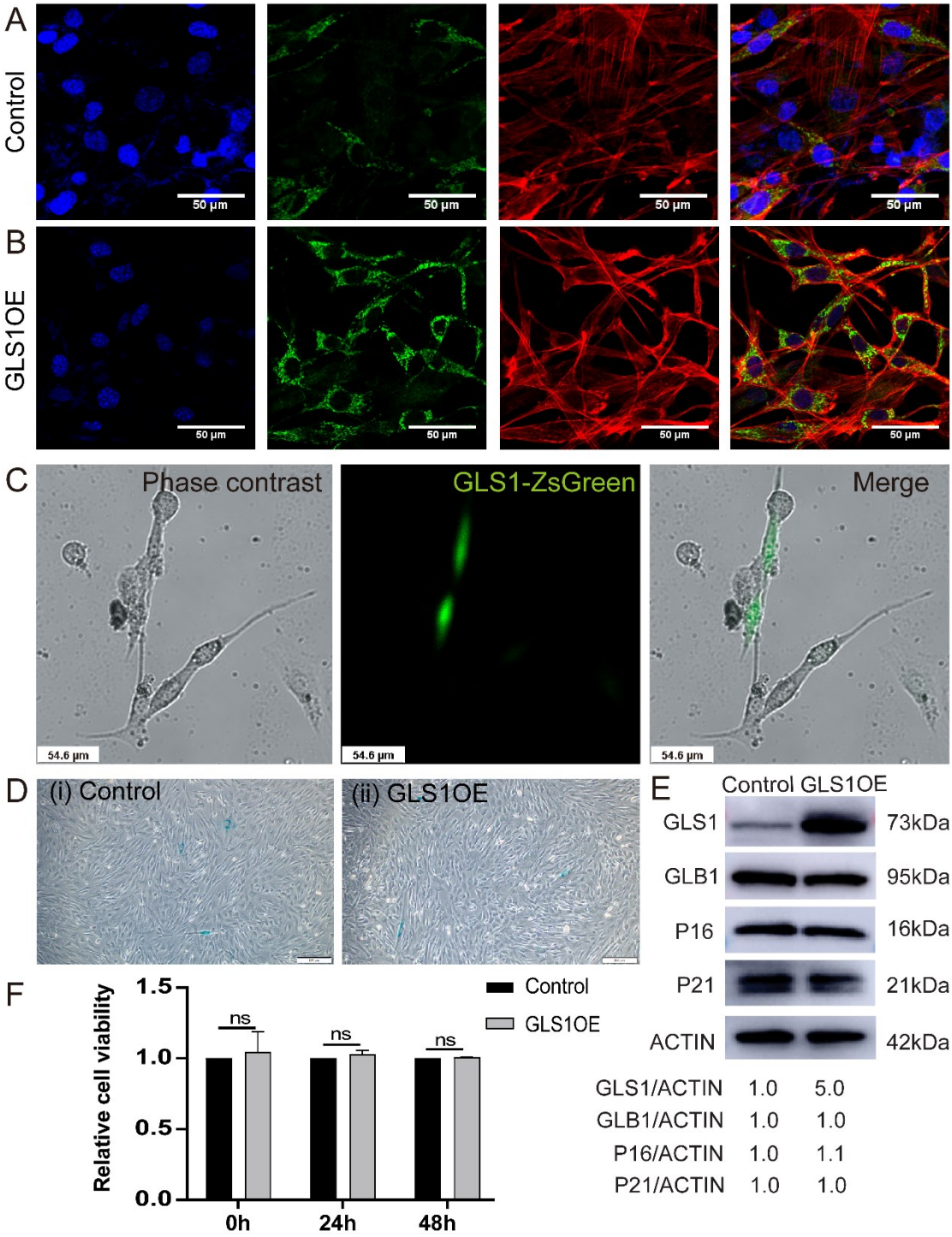

Figure 3: The relationship between GLS1 overexpression and cell senescence. The GLS1 expressed in empty vector transfected cells (A) andpIRES2-GLS1-ZsGreen1 plasmid (B) transfected cells; (C) GLS1 expression after cell dividing; (D) senescence β-Galactosidase staining for control and GLS1 overexpression cells; (E) senescence-related markers in control and GLS1 overexpression groups; (F) cell viability after GLS1 overexpression.

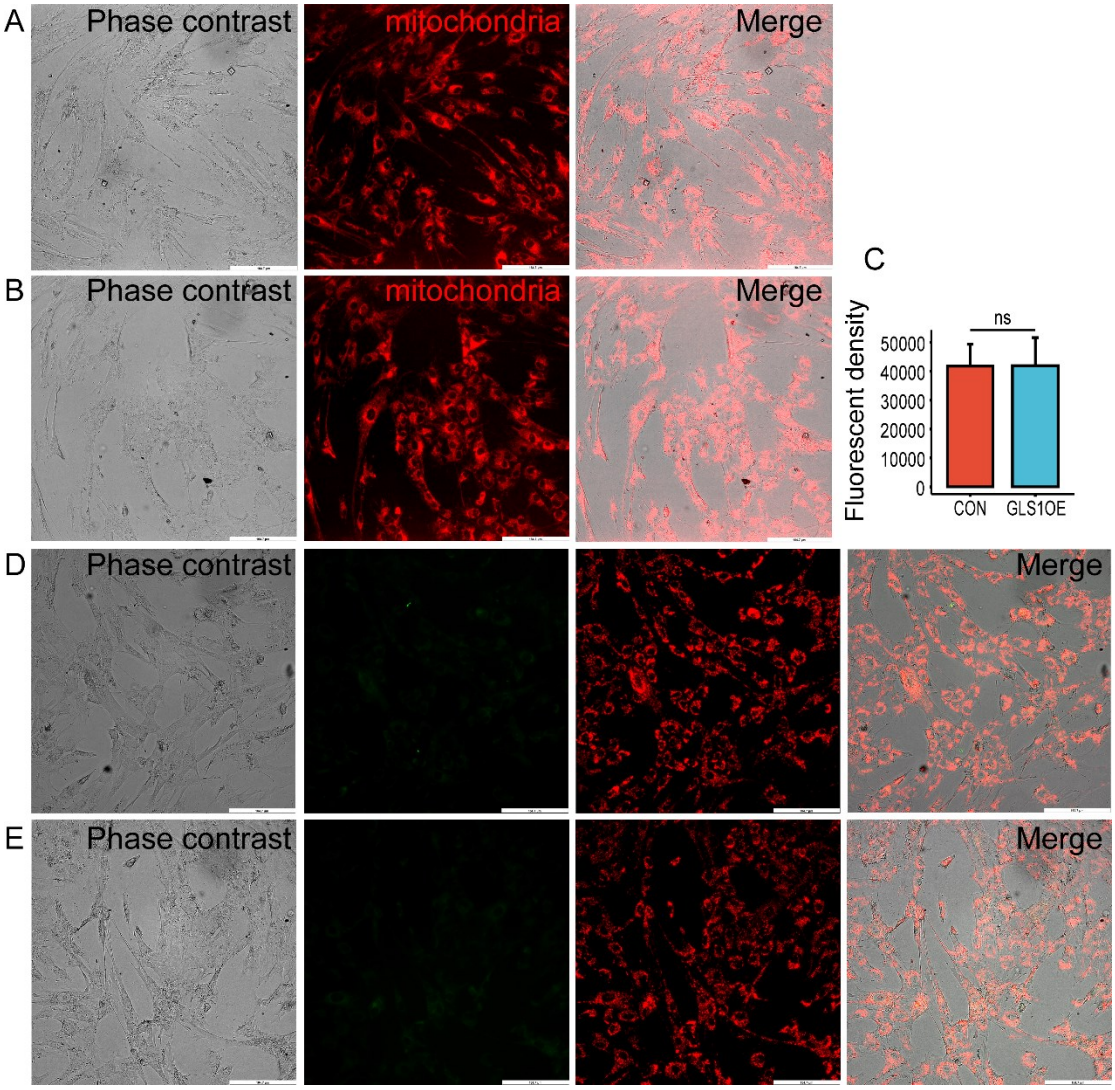

Figure 4: mitochondria function of GLS1 overexpression cells. The live cell mitochondria tracking staining for empty vector transfected cells (A) and GLS1 overexpression cells (B). (C) The fluorescent density of mitochondria staining was calculated by imageJ. The JC-1 assay for empty vector transfected cells (D) and GLS1 overexpression cells (E).

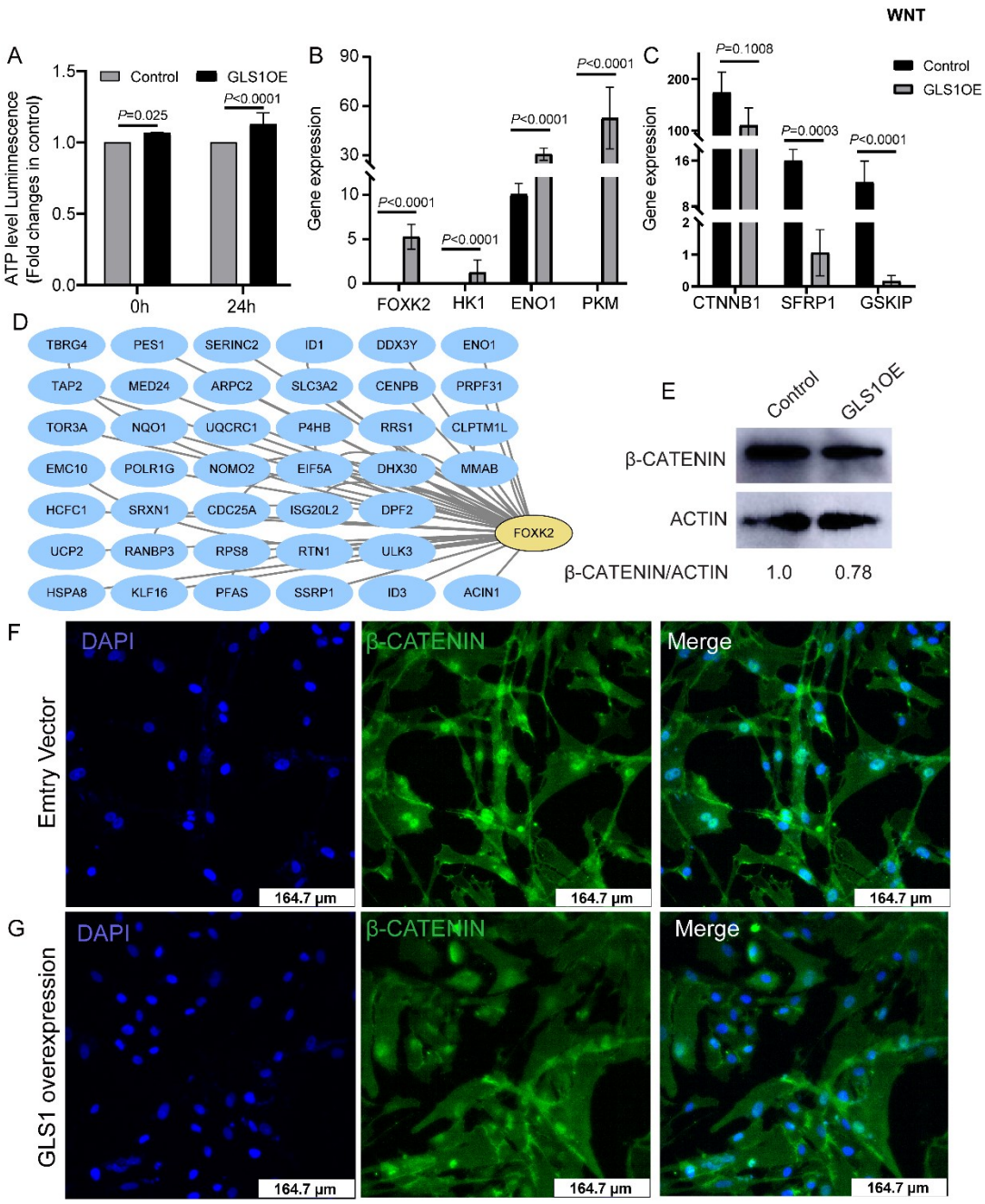

Figure 5: The GLS1 overexpression regulated glycolysis and localization of β-CATENIN via *FOXK2* expression. (A) ATP level in control and GLS1 overexpression cells; (B) mRNA expression of 3 key enzymes of glycolysis; (C) the expression of β-CATENIN-related genes; (D) the expressions of genes positively correlated with *FOXK2* expression in GLS1 overexpression cells; (E) β-CATENIN protein expression in GLS1 overexpression cells; The localization of β-CATENIN in empty vector transfected cells (F) and GLS1 overexpression cells (G).

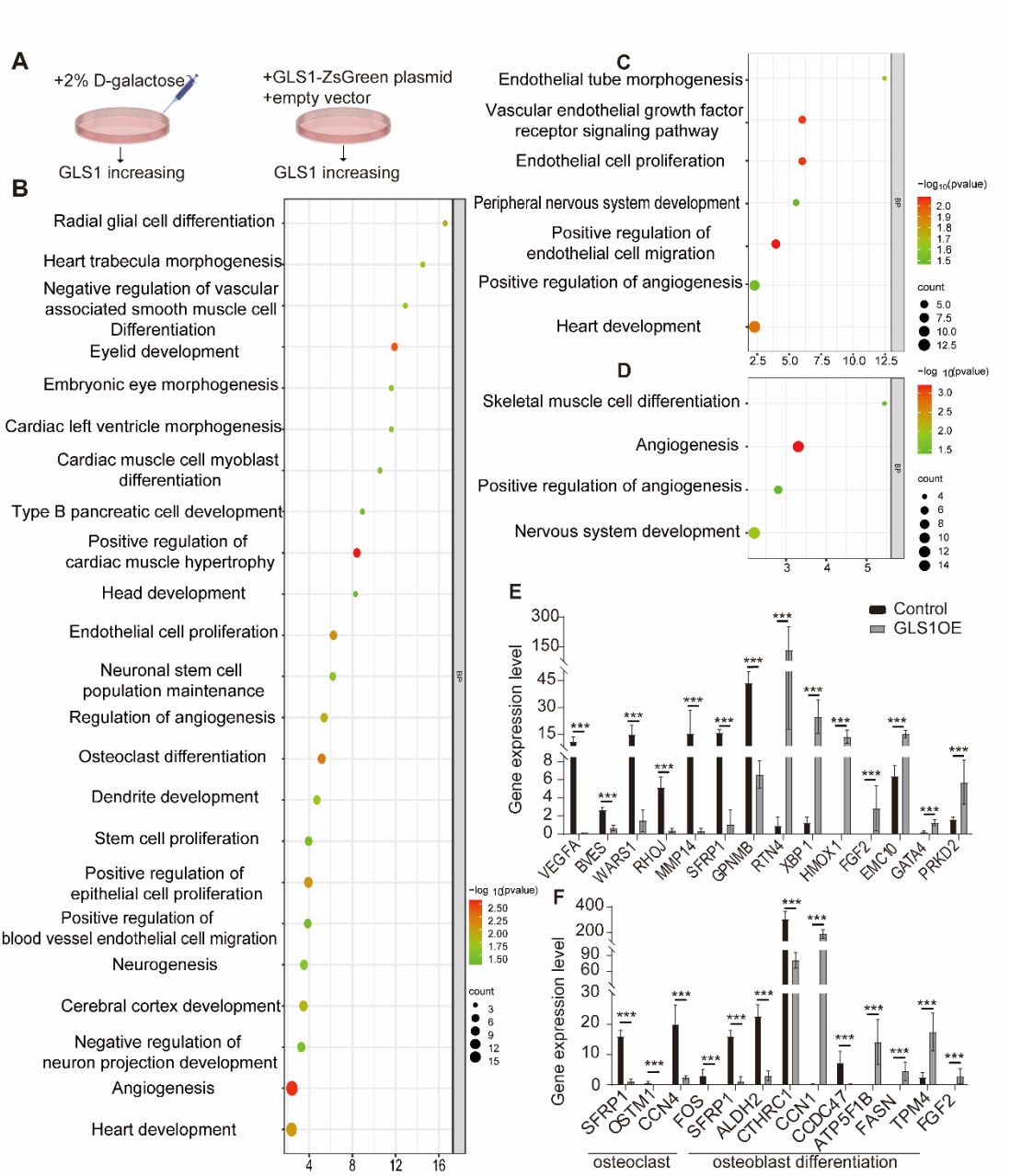

Figure 6： GLS1 overexpression affect the differentiation potential of UCMSCs. (A) Two methods to induce higher GLS1 expression in UCMSCs; The GO enrichment of biological process for normal UCMSCs (B), D-galactose-induced cells (C) and GLS1 overexpression cells (D). The expression level of genes related with angiogenesis (E), osteoclast and osteoblast differentiation (F).